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Axonal precursor miRNAs hitchhike on endosomes and locally regulate the development of neural circuits

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 2nd Jul 19

Thank you for submitting your manuscript on axonal trafficking of pre-miRNAs for consideration by The EMBO Journal. We have now received three referee reports on your study, which are included below for your information.

As you will see, the reviewers are overall positive and acknowledge the in vitro and in vivo analysis you provide. However they also raise some concerns that would need to be addressed in a revised manuscript. In particular, all referees find that the characterization of the trafficking vesicles should be expanded, also with respect to the results of the recent publication by Cioni et al. (Cell 2019), as well as to include a further description of retrograde transport (ref.#1 and #3). In addition, referee #2 points out several experimental controls that should be included to further verify the specificity of the observed effects.

Should you be able to adequately address these key concerns, as well as the other more specific issues raised by each of the referees, then we would be happy to consider this study further for publication. I would therefore like to invite you to prepare and submit a revised manuscript. Please note that it is our policy to allow only a single round of major revision and that it is therefore important to clarify all key concerns raised at this stage.

REFEREE REPORTS

Referee #1:

This manuscript by Corradi et al. describes in an impressive series of very elegant and demanding experiments the mechanism for the inhibition of local protein synthesis in developing retinal

ganglion cell axons. The authors conclusively showed that specific endogenous precursor microRNAs (pre-miRNAs) are actively delivered to growth cones and processed in a Sema3A-dependent manner into active miRNA(s), which silence the basal translation of specific transcripts controlling the tip steering response, such as tubulin beta 3 class III (TUBB3). Crucially, the validation of this mechanism has been performed both in vitro and in vivo, using a assay detecting the anatomical and functional innervation of the visual system.

This manuscript is nicely written and the results are presented in a clear, logical manner in high-quality figures. Quantification and statistical analyses are coupled to representative high-resolution images and well designed schemes, which help the reader through the different experimental approaches.

Few changes and additional experiments may be considered to further refine the conclusion of this excellent work.

1. The initial part of the manuscript describing the delivery of the pre-miRNAs to growth cones is generally the less clear and conclusive of the whole set of results. Whereas the evidence that this is a vectorial, microtubule-dependent process is sufficiently strong, the identity of the organelles contributing to pre-miRNA transport deserves further investigation. In absence of further analyses using bona-fide lysosomal markers (e.g. LAMP1 and 2), and clear evidence that these organelles are degradative and acidic (e.g. lysotracker-positive), it is rather premature to conclude that they are indeed lysosomes. Furthermore, the dynamics of the transport (e.g. balance between anterograde and retrograde flow) does not fit well published data describing the axonal transport of lysosomes.

Whilst these are interesting questions which deserve careful investigation, this reviewer does not feel that addressing them is crucial for publication of this manuscript. However, it would be important that the experiments performed using nocodazole and displaying a rather modest effect on overall puncta speed (Figure 3D), are repeated using a more potent microtubule inhibitor, such as vincristine, to conclusively show that the observed process is strictly microtubule-dependent.

To improve readability, the authors may also consider moving panel F-K of Figure 2 to supplemental data.

2. The in vivo phenotype quantified in Figure 6I is surprisingly very modest when compared with the all-or-none effect detected in vitro, and the axonal mis-projections observed in vivo. Do the authors have any explanation for this weak phenotype?

3. To strengthen the conclusion that this mechanism works in isolated axons, Figure 7I should be completed by adding the effect of WT 3'UTR in the absence and presence of Sema3A.

4. Given the previous literature investigating the role of proteasome activity in growth cone dynamics, it would be worth repeating the key conditions shown in Figure 7H using MG132 or another proteasome inhibitor.

5. A rather obvious questions which seems to have been ignored in the manuscript is the physiological significance of the retrograde pool of pre-miRNAs. Whilst the anterograde function of these pre-miRNAs has been carefully investigated, no working hypothesis for the pool moving in the opposite direction has been provided in the discussion.

Referee #2:

Corradi et al. report their finding that pre-miRNAs are transported into developing axons together with vesicles. Within axons, these pre-miRNAs are converted into functional miRNAs in response to an extracellular signal (Semphorin3A), and they silence the translation of target mRNAs. Interference with the pre-miRNA to miRNA processing in axons causes growth cone collapse defects in vitro and pathfinding abnormalities in vivo.
The presence of non-coding mRNAs including pre-miRNAs and proteins of the RNAi pathway in developing axons is known for over ten years, but their function has remained unclear. The authors'

finding that cue-induced inhibition of the formation of specific miRNAs within axons interferes with axon pathfinding is an exciting new finding and suggests a novel mechanism for the control of local protein synthesis in axons. The first part of the manuscript, describing the transport of pre-miRNA via 'hitchhiking' on vesicles is less novel (compare for example Cioni et al., Cell 2019 reporting that Ribonucleoprotein particles are associated with endosomes in axons). Also, as this is a very crowded manuscript, I am wondering whether the first part, reporting pre-miRNA transport, is necessary or should rather form the basis of a stand-alone manuscript. At the moment, both parts of the manuscript are strangely unconnected (for example, an obvious connection would have been to test whether Sema3A stimulation change transport dynamics of pre-miRNAs).
The experiments are performed to a high standard and overall carefully analyzed. The authors' conclusions are based on in vitro and in vivo approaches, primarily using the Xenopus retinal ganglion model system. There are however a number of missing controls and related issues that ought to be addressed.

Major points:
Lines 89-91 related to Fig. S1B,C: These RT-PCRs are performed with different primer pairs and can not be quantitatively compared. Either perform a qRT-PCR or reformulate.
Line 100 related to Fig. 1F: This figure does not show specificity because it is missing a negative control. The authors should include a negative control (e.g. pre-miR-182).
Line 102 related to Fig. 1G-N: As above, these figures do not address specificity because non-targeted pre-miRs (such as pre-miR-182) are not tested. The authors mistake efficacy for specificity.
Line 115 related to Fig. 2B: Without a quantification of the relative abundance of MB-labeled puncta in distal axons vs. growth cones it is impossible to state that the puncta accumulate.
Lines 153-164 related to Fig. 3E-J: CD63 is an odd choice, because it does not allow to identify the vesicles.
Figure 1-3: As mentioned above, this part of the paper is very phenomenological and adds little to the following figures, which are based on the observation that pre-miRNAs are localized to axons, regardless of how they got here. If they authors want to keep the figures as part of this manuscript, they should test whether transport dynamics are affected by Semaphorin3A treatment, and they should identify the vesicles further using specific markers for LE, lysosomes, MVBs, etc.
Line 179 related to Fig 4D: The HA- and neurofilament signal overlap only very little. Most if not all HA-Dicer seems to be outside of axons. A higher magnification and co-localization analysis would be necessary to support the authors' claim of clear overlapping signals.
Lines 193-197 related to Figs. 5B,C: the authors should check for mir-182-5p and -3p levels as negative controls.
Lines 251-253: The argument against mRNA degradation based on the time frame is weak. The authors should measure target mRNA levels by qRT-PCR if they want to make this claim.
Figure 7: This entire figure is using overexpressed reporter constructs as proxies for the translation of endogenous mRNA. The authors' main finding of the paper is that local pre-miRNA processing shifts the balance between mRNA and miRNAs locally (see scheme). The overexpression of the reporter constructs is likely to affect this balance as well, confounding the interpretation of these data. Instead of using exogenous report constructs, it would be much stronger and more reliable to measure the translation of endogenous target mRNAs of miR-181a directly. There are several methods such as puro-PLA or BONCAT that can be used together with the MOs.
How many localized mRNAs are likely regulated by miR-181a? Does delivery of siRNA against TUBB3 rescue the growth cone collapse phenotype seen in Fig. 5E,F or in other words?

Minor Points:
Lines 23, 58 - what is the meaning of delocalization? From the context it should be localization.
Line 49 - use 'and' instead of 'but', as the sentence does not state a contradiction.
Line 88 - reformulate half-sentence following 'and'.
Line 136 - remove promptly.
Line 160 - Spelling: co-traffic.
Line 207 - 6A should be S6A.

Referee #3:

The study emerged from earlier intriguing findings that besides mRNAs also other RNAs like non-

coding RNAs, miRNAs etc are also found enriched in specific subcellular outposts, but the underlying mechanism remained elusive until now. The authors first convincingly show, using optimized procedures with molecular beacons, that precursor pre-miRNAs appear to be actively transported to the growth cone central area by hitchhiking on CD63-positive late endosomal organelles along the axons. These findings extend a recent study where mRNAs use endosomal compartments for transport and as organellar translation hubs. Next, they demonstrate that in the growth cone, these pre-miRNAs are processed into active miRNAs and that this occurs in a response to repressive cues. Finally, they identify one of the targets, TUBB3, that is silenced through specific miRNAs when neurons are exposed to Sema3A. Overall, the authors describe a novel mechanism by which neuronal outgrowth and remodelling can be modulated through local activation of specific miRNAs in response to cues. Moreover, they provide important in vivo support for this mechanism, upkeeping a new additional regulatory layer in brain connectivity. Thus, external cues can activate parallel pathways regulating the expression of separate growth cone proteins, on through eliciting a local increase in protein synthesis, while another one inhibits protein synthesis (as an alternative to local protein degradation). This allows for a fine-tuned balance in axon remodelling during brain development.
The experiments, both in vitro and in vivo, are to my opinion well-controlled and convincing. The authors did overcome several technical hurdles to uniquely detect premiRNAs, implemented a broad range of approaches and controls that brought together an impressive set of original data. To my opinion, the originality and wealth of data support publication in EMBO Journal.
I do not have major concerns, but rather some reflections that may require some more explanation or control experiments.

Minor concerns:
- PremiRNAs traffick retrogradely and anterogradely with the same frequencies. Why are premiRNAs retrogradely transported and is there like the central region in the growth cone also a region in the retrograde direction (cell body?) where they concentrate (and may become activated as well)?
- Fig. 3B,C: treatment with nocodazole increases the proportion of stationary puncta while an overall decrease of average velocities of puncta. When comparing this set of data with figure 2, the proportion of actively moving puncta is by far the major population in panel E and H, while in fig 3, it is roughly similar in proportion to the confined puncta (red bars in fig 3C). Do the authors have an explanation for this as they claim that most premiRNA are actively transported along MTs.
- Related to figure 3 the authors indicate that mRNAs are trafficked along axons within RNPs, while data indicate that miRNAs (and other related RNAs) associate with LE/MVBs. Therefore, the authors reasoned that premiRNAs may use LE/MVBs to traffick along axons. However, recently (Cione et al., 2019) mRNAs were found to be associated with endosomal organelles (not limited to LE/MVBs) as well during transport. It would be interesting to demonstrate whether the same or distinct populations of LE/MVBs are used for both cargo's. Likewise, the authors cannot exclude that other organelles besides LE/MVBs are involved: a co-staining with other endosomal organelles (EEA1-/Rab5-GFP, Lamp1-GFP) should be performed to demonstrate unique associations.
To conclude the authors show that in the growth cone Sema3A induces an activation cascade generating local miRNAs that silence specific targets such as TUBB3 resulting in MT remodelling and finally growth cone collapse. The immediate next and important question is how Sema3A signals to the machinery activating local premiRNAs. Although this requires a new set of experiments beyond the scope of the current manuscript, can the authors speculate a bit more and include their thoughts in the discussion section?

1st Revision - authors' response 8th Nov 19

The Editor:

“all referees find that the characterization of the trafficking vesicles should be expanded, also with respect to the results of the recent publication by Cioni et al. (Cell 2019), as well as to include a further description of retrograde transport (ref.#1 and #3). In addition, referee #2 points out several

experimental controls that should be included to further verify the specificity of the observed effects."

AUTHORS' REPLY: We have addressed below these three key points.

1. Characterization of the trafficking vesicles (all Reviewers.)

We have used a wide panel of markers to further identify the vesicles to which pre-miRNAs appear associated. Our revised and complete analysis suggests that pre-miR-181a-1 is trafficked primarily docked to late endosomes / lysosomes. These new results are shown in Fig 4A-I and Appendix Fig S3A-G, and described in the results section in page 7 and 8 (lines 181-203).

Following the suggestions of the Reviewers, we have used similar markers as the one published in Cioni et al. (cell, 2019) namely Rab5a, Rab7a and Lamp1, as they are enriched in early, late endosomes and lysosomes, respectively (Huotari & Heleniun, 2011, EMBO J; Saftig & Klumperman, 2009, Nature reviews; Langemeyer et al. 2018, Trends Cell Biol.). It is important to stress that no markers are specific for a particular stage of endosome maturation (Cheng et al. 2018, JCB ; Yap et al. 2018, JCB ; Von Bartheld & Altick 2011 Prog Neurobiol). For instance in neurons, Rab5a (enriched in early endosomes) and Lamp1 (enriched in lysosomes) can also be found in maturing early late endosomes and pre-degradative late endosomes, respectively (Yap et al., 2018, JCB). Our key results are summarized below.

We have first characterized our CD63 marker and found that it highly colocalized with Rab7a (81%), Lamp1 (81%), but significantly less with Rab5a (39%). In addition, LysoTracker, a marker of acidic organelles, colocalized with CD63 (76%), Rab7a (80%) and Lamp1 (83%). This suggests that the CD63 marker that we have used to study pre-miRNA co-trafficking is primarily present in acidic late endosomes / lysosomes in embryonic *Xenopus* RGC axons. Pre-miR-181a-1 highly colocalized with CD63 (80%) as well as with the late endosomal and lysosomal markers Rab7a (90%), LAMP1 (91%), and lysotracker (90%), and vesicles marked by both CD63/lysotracker (68.5%) and CD63/Rab7a (80%). Conversely, its association with the early endosomal marker Rab5a was significantly lower (50%). Collectively, these data suggest that pre-miRNAs are primarily transported by late endosomes / lysosomes.

To complete our analysis and although not requested, we have also performed 3D-STED super resolution microscopy. We deemed that these experiments were important to rule out the possibility that the exogenous cy3-pre-miR-181a-1 and cy3-MB are incorporated inside intraluminal vesicles of late endosomes / lysosomes for degradation or exocytosis. We detected that all of the 390 exogenous and endogenous pre-miR-181-a1 puncta analyzed were associated with the outer surface of CD63-GFP-marked vesicles. Collectively, our data suggest that pre-miRNAs are docked to the outer surface of endosomes for transport, readily available for cytoplasmic function. These new results are shown in Fig 4I and Appendix Fig S3G, and described in the results section in page 8 (lines 197-203).

Together, the new results obtained in this revised manuscript confirm our initial findings and further identify late endosomes / lysosomes as the main vesicular compartment carrying pre-miR181a in axons.

2. Further description of the retrograde transport (Reviewer #1 and #3)

Whilst Dicer localization at the growth cone suggests that pre-miRNAs would be primarily if not only transported anterogradely to reach the distal tip of the axon for processing, we do observe that an equal number of puncta are retrogradely transported.

We have developed our views below on the various physiological mechanisms that could be supported by the retrograde transport of a pool of pre-miRNAs. These ideas have been included in our revised manuscript and can be found on page 17 (lines 435-446).

Anterograde and retrograde directed transport of mRNAs have been observed in both dendrites and axons. In dendrites, a sushi-belt model of mRNA localization has been proposed whereby mRNAs are transported bidirectionally to patrol through dendritic spines until they are required for translation (Doyle and Kiebler, 2011, EMBO J). Similarly, we can speculate that pre-miRNAs are scanning the axons until they are required for mRNA silencing. It is thus interesting that we observe an increase in static puncta upon Sema3A stimulation. Where could translation silencing occur besides the growth cone? While we clearly detect that NGmiRNAs modulate axon targeting, it is possible that they also regulate branching, a process that occurs soon after RGC axons reach the target and that can be observed *ex vivo* upon Sema3A stimulation (Campbell et al., 2001, J of neurosci). Pre-miRNAs might be surveilling new branching points along the axons and be recruited there to modulate the translation of TUBB3 and other targets similarly to the proposed model of "patrol and local entrapment" (Das et al., 2019, Curr opinion in Neurobiol).

Retrograde transport could also be a means to monitor the copy number of pre-miRNAs that are present within the growth cones. miRNA-mediated silencing is a process that requires fine-tuned stoichiometry between a pool of mature miRNAs and a pool of mRNA targets to insure the production of an exact copy number of newly synthesized proteins (Bartel, 2008, Cell). It is possible that pre-miRNAs are retrogradely transported because the adequate numbers of molecules are already stored at the growth cone in basal conditions and any extra copy needs to be sent back.

Furthermore, retrogradely transported pre-miRNAs could act as a long-range intracellular messenger and inform the soma of events occurring in the cell periphery such as the axonal distal tip. What kind of information could be provided? Retrograde pre-miRNAs could be processed by the soma to modulate its own production by targeting its transcription regulators through a negative feedback loop. It could also target somatic transcripts as a response to a stimulus

received at the periphery. This could be the case for instance when developmental transitions are required and somatic mRNAs supporting one stage of development (e.g. targeting) need to be cleared to facilitate the transition to the following stage (e.g. branching). In this scenario, the periphery would be informing the soma that a stage has been completed (e.g. the growth cone has reached its target and is ready to branch). The roles of miRNAs in such developmental transitions are well described (Ambros et al., 2011, Curr Opin Genet Dev).

3. Additional control experiments (Reviewer #2)

We have performed additional experiments to verify the specificity of the observed effects, as requested by Reviewer 2. We have also added one set of experiments not originally requested. Detailed answers to Reviewer 2 and revised manuscript line numbers can be found in our point-by-point reply to the Reviewers Section.

3.1. Specificity of the MB *in vitro*

We have first addressed whether the MB binds specifically to its target by thermal denaturation using not only a pre-miRNA other than pre-miR-181a-1, as suggested by the Reviewer, but also two additional controls. Our new analysis clearly shows that the MB opens up at lower temperature in the presence of pre-miR-181a-1 but does not do so with any of these additional three controls (Appendix Fig S1D-F). This result demonstrates that the MB used in this study specifically binds to the loop region of pre-miR-181a-1.

3.2. Specificity of the MB *ex vivo*

As suggested by Reviewer No2, we have improved our initial analysis showing the specificity *ex vivo* of the MB used in this study (Fig 1G-N). For this, we have performed new experiments using an additional pre-miRNA control that is not recognized by the MB through thermal denaturation. Our new results show very low colocalization between the MB and this exogenous control pre-miRNA (Appendix Fig S1G-L) strengthening the notion that the MB specifically detects pre-miR-181a-1.

3.3. Specificity of pre-miRNA biogenesis

We have consolidated our data set showing that pre-miR-181a-1 is specifically processed by Sema3A by analyzing the levels of miR-182-5p and -3p (Appendix Fig S4D,E). We find that Sema3A does not alter the levels of these mature miRNAs, confirming that this cue triggers the biogenesis of specific miRNAs.

3.4. Specificity of Sema3A in eliciting NGmiRNA-mediated collapse response

Although this was not requested, we have also assessed whether pre-miRNA processing specifically impinge on Sema3A signaling pathway. For this, we have tested an additional guidance cue (Slit2) which does not alter pre-miRNA processing and found no change in mature miRNA levels with or without Dicer cleavage blocking MO. We have modified the Supplementary figure (new Fig. EV4G) and the results (page 10 (lines 268-269)), accordingly.

We have next answered the Reviewers comments point-by-point.

Reviewer #1:

This manuscript by Corradi et al. describes in an impressive series of very elegant and demanding experiments the mechanism for the inhibition of local protein synthesis in developing retinal ganglion cell axons. The authors conclusively showed that specific endogenous precursor microRNAs (pre-miRNAs) are actively delivered to growth cones and processed in a Sema3A-dependent manner into active miRNA(s), which silence the basal translation of specific transcripts controlling the tip steering response, such as tubulin beta 3 class III (TUBB3). Crucially, the validation of this mechanism has been performed both in vitro and in vivo, using a assay detecting the anatomical and functional innervation of the visual system.

This manuscript is nicely written and the results are presented in a clear, logical manner in high-quality figures. Quantification and statistical analyses are coupled to representative high-resolution images and well designed schemes, which help the reader through the different experimental approaches.

Few changes and additional experiments may be considered to further refine the conclusion of this excellent work.

AUTHORS' REPLY: We thank the Reviewer for the insightful comments and criticisms that contributed to improve the quality of our work, and for pointing out that our work "describes in an impressive series of very elegant and demanding experiments" and that the "manuscript is nicely written and the results are presented in a clear, logical manner in high-quality figures. "

1apartI The initial part of the manuscript describing the delivery of the pre-miRNAs to growth cones is generally the less clear and conclusive of the whole set of results. Whereas the evidence that this is a vectorial, microtubule-dependent process is sufficiently strong, the identity of the organelles contributing to pre-miRNA transport deserves further investigation. In absence of further analyses using bona-fide lysosomal markers (e.g. Lamp1and 2), and clear evidence that these organelles are degradative and acidic (e.g. LysoTracker-positive), it is rather premature to conclude that they are indeed lysosomes.

AUTHORS' REPLY: We thank the Reviewer for this comment. We have further characterized the identity of the organelles contributing to pre-miRNA transport using Lamp1 and LysoTracker as markers of lysosomes and degradative organelles, respectively.
We have also used additional makers, Rab5a and Rab7a. Our complete analysis is detailed in our answer to the Editor 's first point in page 1 and 2 above.

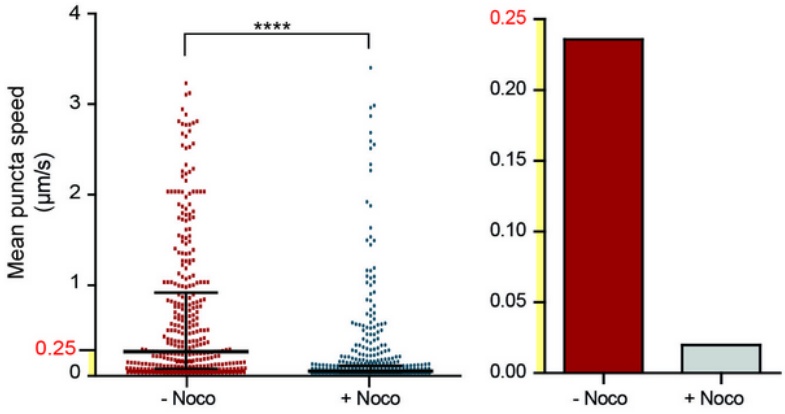
Collectively, our new data suggest that pre-miRNAs are primarily transported by late endosomes / lysosomes. These data are shown in the Fig 4 and Appendix Fig S3, and described in the result section in page 7 and 8 (lines 181-196).

1apartII Furthermore, the dynamics of the transport (e.g. balance between anterograde and retrograde flow) does not fit well published data describing the axonal transport of lysosomes.

AUTHORS' REPLY: Early investigations in adult axons have led to a model whereby axonal lysosomes are primarily retrogradely transported towards the cell body (Ferguson et al., 2018, Curr. Opinion in Neurobiology). Recent studies have, however, revealed a balanced transport in both directions when specifically looking at live trafficking of late endosomes / lysosomes in developing axons. Farias et al. (2018, PNAS), for instance, thoroughly investigated late endosome / lysosome transport in axons and dendrites of rat E18 embryonic hippocampal neurons using live-cell imaging and detected that Lamp1-GFP-marked motile endosomes were moving bidirectionally (50.4% anterograde and 49.6% retrograde) in axons and showed a comovement with CD63-GFP. The result section has been modified in page 6 (lines 169-170) to compare our results with this study. Furthermore, Cioni and colleagues (2019, Cell) have also detected a balanced transport of LysoTracker-positive vesicles in *Xenopus* embryonic RGCs. The fact that we detect a balanced pre-miRNA transport associated with CD63-GFP marker is thus in line with the recent literature focusing on embryonic neurons.

1b. Whilst these are interesting questions which deserve careful investigation, this Reviewer does not feel that addressing them is crucial for publication of this manuscript. However, it would be important that the experiments performed using nocodazole and displaying a rather modest effect on overall puncta speed (Figure 3D), are repeated using a more potent microtubule inhibitor, such as vincristine, to conclusively show that the observed process is strictly microtubule-dependent.

AUTHORS' REPLY: We agree with the Reviewer that the layout of the former Fig 3D (now Fig 2L) does not convey a strong effect on overall puncta speed. We show below another representation.



Following nocodazole treatment, the median puncta speed significantly slowed down to almost a complete stall from 0.2357 um/s to 0.0199 um/s (median speeds). This represents a 91.5% reduction in median velocity which we believe is very strong and within the range of what others have published with similar experimental approaches (see for instance Leung et al., 2018, Front Cell Neurosci, who observed a reduction of exogenous cy3-labeled beta-actin speed 67% down to 0.15µm/s with nocodazole application to *Xenopus* RGC axons). To fully illustrate the *bona fide* strength of the effect, we have specified in the text the values of the median speed and the extent in the speed reduction in page 6 (lines 151-154).

Nevertheless, as suggested by the Reviewer, we have performed additional experiments using Vincristine and we observed a 94.4% reduction in median speed and an increase in proportion of the stationary (<0.2µm/s) puncta at the expense of fast moving (>0.5µm/s) puncta, similar to what was observed for Nocodazole treatment. We have now modified the text to include these new data in page 6 (lines 154-155), and inserted the data in Fig EV1D and E.

1c.To improve readability, the authors may also consider moving panel F-K of Figure 2 to supplemental data.

AUTHORS' REPLY: Following the Reviewer's suggestion, we have reorganized Fig. 2 to improve readability.

2. The in vivo phenotype quantified in Figure 6I is surprisingly very modest when compared with the all-or-none effect detected in vitro, and the axonal mis-projections observed in vivo. Do the authors have any explanation for this weak phenotype?

AUTHORS' REPLY: We agree with the Reviewer. The *in vivo* behavioural phenotype quantified in former Fig 6I (new Fig. 7I) is indeed very modest when

compared with axonal mis-projections observed *in vivo* in former Fig 6B and C (new Fig 7B and C) and Sema3A-induced collapsed growth cones *ex vivo* in former Fig 5E and F (new Fig 6E and F). As developed below, we attribute this lack of consistency mostly due to the differing types of axons under investigation. Wildtype axons are indirectly included in behavioural studies, likely lessening the impact of the effect of miR-181 LOF, whilst only morphant axons are counted in the two other analyses.

In both the behavioural study in former Fig 6I and *in vivo* misprojection analysis in former Fig. 6F,G, MOs are targeted at stage 26 and biological read-out performed at stage 44/45. In these conditions, about 75-100% of RGCs and progenitor cells fated to become RGCs are targeted with morpholinos in our hands. As the eye develops and new cells are born past the electroporation stage, the proportion of targeted cells vs total retinal cells decreases. Furthermore, morpholinos are also cleared out. Overall, we estimate that 30-50% RGCs will contain the MO at the critical stage of circuit formation including targeting, and the visual pathway will be composed of a mix of morpholino-targeted and wildtype RGC axons.

In behavioural studies, we measure the % of time each embryo spent on the black background as a proxy of visual processing from all axons, targeted by the morpholinos ("morphant") and non-targeted (wildtype). In *in vivo* axon misprojection analysis, on the other hand, we measure the % of misguided targeted axons compared to the total number of targeted axons, not to the total number of axons composing the visual pathway. So in this latter case, the phenotype is analyzed on all targeted morphant axons. Similarly, *ex vivo* analysis is performed on morphant axons: on cultured axons transfected with morpholinos. The presence of a considerable number of wildtype axons in the behavioural analysis would significantly lessen the extent of the effect observed and is likely to explain the much milder phenotype detected and the different results obtained compared to *in vivo* and *ex vivo* analyses.

It is important to specify, however, that we are actually measuring radically different output: % of time the embryos spent on black background (behavioural studies) versus % of misprojecting targeted axons normalized to total targeted axons (*in vivo*) versus % of collapsed growth cones from transfected axons (*ex vivo*). Strictly speaking, these biological read-outs cannot be compared and one thus expect different phenotype penetrance compared to control.

Finally, we would like to stress that our results obtained in former Fig 6I (new Fig 7I), are in line with published data showing that knockout of most individual miRNAs, contrary to protein coding genes, do not result in grossly abnormal phenotypes *in vivo* (for instance see Miska et al., 2007, PLOS Genetics; Ebert and Sharp, 2012, Cell). Individual miRNAs are tightly integrated in complex cellular networks, and their functions are best highlighted by analyzing individual miRNA knockouts in sensitized genetic backgrounds (Brenner JL, et al., 2010, Curr Biol) or under stress (Rasmussen, et al., 2010, J Exp Med). Overall miRNAs are acknowledged to have subtle and redundant roles, which is consistent with our present results.

3. To strengthen the conclusion that this mechanism works in isolated axons, Figure 7I should be completed by adding the effect of WT 3'UTR in the absence and presence of Sema3A.

AUTHORS' REPLY: We agree with the Reviewer that performing additional experiments in isolated axons using WT 3'UTR in the absence and presence of Sema3A would be of interest and strengthen the current dataset. However, these experiments are particularly challenging and time consuming to conduct, because it is difficult to maintain the health of severed axons following the bleaching with the high energy UV light required in FRAP. For this reason, we had primarily focused on non-isolated axons where we performed an entire set of experiments with a wide panel of associated controls (including WT 3'UTR in the absence and presence of Sema3A) and repeated the key experiment in isolated axons. In isolated axons, we had examined the fluorescence recovery of Venus under the translational control of TUBB3-MUT 3'UTR and found a 10 min-level of fluorescent recovery around 23.90% +/-3.66 -Sema3A; 22.52% +/- 5.83 +Sema3A, which was very similar to that found in non-isolated axons (24.14% +/- 2.24 -Sema3A; 23.15% +/- 3.08 +Sema3A). Data generated in isolated and non-isolated axons were therefore very consistent and suggested that the regulation that we observed occurs locally without the influence from the soma.

In addition, we had also showed that a similar effect occurs in isolated axons with a different approach. Using Dicer cleavage blocking MOs-3p, we were able to detect a 10 min-level of Venus-TUBB3-WT fluorescent recovery around 26.1972% +/- 4.817541+Sema3A (compared to co-MO control: 12.98198% +/- 2.165362+Sema3A). These results were similar to that found in non-isolated axons and isolated axons using the Venus-TUBB3-MUT 3'UTR reporter (isolated: 22.52% +/- 5.83 +Sema3A; non-isolated: 23.15% +/- 3.08 +Sema3A). Furthermore, we have now also reproduced these key findings in isolated axons using an entirely different technique. Using puro-PLA, we detect that blocking pre-miRNA processing with MOs-3p abolishes the effect of Sema3A on **endogenous** TUBB3 silencing, confirming the results obtained using Venus-TUBB3 3'UTR as an exogenous translational reporter. These new data are now shown in Fig 9A-E, and described in the result section in page 14 (lines 358-366).

Collectively, these four sets of experiments enable us to confidently conclude that Sema3A-induced miR-181 activation and function is critical in TUBB3 translational inhibition locally within axons.

4. Given the previous literature investigating the role of proteasome activity in growth cone dynamics, it would be worth repeating the key conditions shown in Figure 7H using MG132 or another proteasome inhibitor.

AUTHORS' REPLY: Investigating the role of proteasome activity in growth cone dynamics would be interesting. However we believe that this is beyond the scope of our study as it mostly focuses on the canonical roles of miRNA in translational silencing. We expect that the use of proteasome inhibitor will not modulate basal or Sema3A-induced TUBB3 translation inhibition examined in former Fig 7H (new

Fig 8H), since proteasome mediated proteolysis is not elicited in basal conditions, and since Sema3A does not induce such proteasome activity in *Xenopus RGC* (Campbell and Holt, 2001, Neuron).

5. A rather obvious questions which seems to have been ignored in the manuscript is the physiological significance of the retrograde pool of pre-miRNAs. Whilst the anterograde function of these pre-miRNAs has been carefully investigated, no working hypothesis for the pool moving in the opposite direction has been provided in the discussion.

AUTHORS' REPLY: we thank the Reviewer for this suggestion. We have now addressed in our answer to the Editor (point 2 above page 2 and 3) our ideas on the possible physiological significance of the retrograde pool of pre-miRNAs and summarized our thoughts in the discussion in page 17 (line 435-446).

Reviewer #2:

Corradi et al. report their finding that pre-miRNAs are transported into developing axons together with vesicles. Within axons, these pre-miRNAs are converted into functional miRNAs in response to an extracellular signal (Semphorin3A), and they silence the translation of target mRNAs. Interference with the pre-miRNA to miRNA processing in axons causes growth cone collapse defects in vitro and pathfinding abnormalities in vivo.
The presence of non-coding mRNAs including pre-miRNAs and proteins of the RNAi pathway in developing axons is known for over ten years, but their function has remained unclear. The authors' finding that cue-induced inhibition of the formation of specific miRNAs within axons interferes with axon pathfinding is an exciting new finding and suggests a novel mechanism for the control of local protein synthesis in axons. The first part of the manuscript, describing the transport of pre-miRNA via 'hitchhiking' on vesicles is less novel (compare for example Cioni et al., Cell 2019 reporting that Ribonucleoprotein particles are associated with endosomes in axons). Also, as this is a very crowded manuscript, I am wondering whether the first part, reporting pre-miRNA transport, is necessary or should rather form the basis of a stand-alone manuscript. At the moment, both parts of the manuscript are strangely unconnected (for example, an obvious connection would have been to test whether Sema3A stimulation change transport dynamics of pre-miRNAs).
The experiments are performed to a high standard and overall carefully analyzed. The authors' conclusions are based on in vitro and in vivo approaches, primarily using the Xenopus retinal ganglion model system. There are however a number of missing controls and related issues that ought to be addressed.

AUTHORS' REPLY: We thank the Reviewer for the insightful comments and criticisms that contributed to improve the quality of our work, and for pointing out that our study provides "exciting new finding" and "are performed to a high standard and overall carefully analyzed"

Major points:

1. Lines 89-91 related to Fig. S1B,C: These RT-PCRs are performed with different primer pairs and can not be quantitatively compared. Either perform a qRT-PCR or reformulate.

AUTHORS' REPLY: We thank the Reviewer for this comment. We agree that our original text in lines 89-91 related to Appendix Fig S1B,C was not clear and has been reformulated. As developed below, the results shown in former Fig.1B and C are derived from RT-qPCR and not RT-PCR.

In the section mentioned by the Reviewer, we wanted to appreciate the relative levels of pre-miR-181a-1 vs pre-miR-181a-2 within given tissues: eye and especially in isolated axons. Our ultimate goal was to understand which of the two isoforms was the most abundant in axons to subsequently focus on it for trafficking analysis. For this, we did perform qPCR using an appropriate reference gene to be able to compare the levels of pre-miR-181a-1 and -2. Our RT-qPCR results revealed that pre-miR-181a-1 was 2.5X more abundant in isolated axons.

We have now modified the results to clarify this point in the main text in page 3 and 4 (lines 92-95).

2.Line 100 related to Fig. 1F: This figure does not show specificity because it is missing a negative control. The authors should include a negative control (e.g. pre-miR-182).

AUTHORS' REPLY: To address this important comment, we have performed new experiments to include additional controls. We have opted to use pre-miR-187 (see answer to point 3 below for rationale). We have also used two additional controls, namely 1) a modified version of pre-miR-181a-1 that does not contain the loop region and 2) a miRNA mimic that contains only the hybridized 3' and 5' mature miRNAs. Since the MB is predicted to target the pre-miR-181a-1 loop, none of these controls should in principle lead to the opening of MB, the dequenching of cy3 and the production of fluorescent signal at low temperature. Our results are fully in line with this prediction.

Our new analysis clearly shows that the MB opens up at lower temperature in the presence of pre-miR-181a-1 but does not do so with any of these additional three controls. This demonstrates that the MB is specific for the loop region of pre-miR-181a-1.

These results are now shown in Appendix Fig S1D-F and described in the results' section (page 4 (lines 102-107) and discussion (page 15 (lines 398-400).

3.Line 102 related to Fig. 1G-N: As above, these figures do not address specificity because non-targeted pre-miRs (such as pre-miR-182) are not tested. The authors mistake efficacy for specificity.

AUTHORS' REPLY: We think that Fig1G-J is addressing the issue of specificity, besides that of efficacy, because the MB loses its ability to detect pre-miR-181-a1 *ex vivo* when this MB targets are knocked down. If the MB was not specifically targeting pre-miR-181a-1 in axon, the MB would be detected to a similar extent whether or not the levels of these molecules are altered.

Nevertheless, we have added a negative control to Fig K-N as suggested by the Reviewer. The addition *ex vivo* of exogenous fluorescently labeled molecules at doses high enough to be imaged is likely to create artifacts. To limit such confounding factor, we have selected a negative pre-miRNA control normally absent from *Xenopus* RGC axons (this study and Bellon et al., 2017, Cell Reports), namely pre-miR-187. We have assessed whether the MB recognizes exogenous cy5-labeled pre-miR-187 with a similar experimental paradigm as that shown in Fig 1K. Our new results show that the MB colocalizes with 2.9% exogenous pre-miR-187-cy5 compared to 77.2% exogenous pre-miR-181a-1.

These results are now shown in Appendix Fig S1G-L and described in the results' section (page 4 (lines 115-116)) and the discussion (page 15 (lines 400-401)).

4.Line 115 related to Fig. 2B: Without a quantification of the relative abundance of MB-labeled puncta in distal axons vs. growth cones it is impossible to state that the puncta accumulate.

AUTHORS' REPLY: To evaluate the relative abundance of MB-labeled puncta in distal axons vs growth cones, we have performed quantitative immunofluorescence (QIF). We focused on the central domain of the growth cone, were puncta appear to accumulate. We didn't count the number of puncta *per se* because their density in the growth cones is too high and not amenable to distinguish them and therefore to perform accurate measurements. Our QIF analysis reveals that MB- and CD63-associated fluorescence is higher in the central domain of the growth cone than in the adjacent axon shaft.

These results are now shown in Fig 3J-L and Appendix S2H,I and described in the results' section (page 7 (lines 174-177).

5.Lines 153-164 related to Fig. 3E-J: CD63 is an odd choice, because it does not allow to identify the vesicles.

AUTHORS' REPLY: We selected CD63 as a marker because it is a transmembrane protein that is enriched in late endosomes / multivesicular bodies and lysosomes (Pols & Klumperman, 2009, Experimental Cell Research). This marker has also been used in other publications to trace late endosome / lysosome trafficking in axons among other markers (see for instance Farías et al., 2017, PNAS). We have, however, selected a panel of additional markers, namely LysoTracker, Lamp1,

Rab7a and Rab5a, to identify the nature of the vesicles on which pre-miRNA is hitchhiking. Our complete analysis is detailed in our answer to the Editor ’s first point above in page 1-2.

6.Figure 1-3: As mentioned above, this part of the paper is very phenomenological and adds little to the following figures, which are based on the observation that pre-miRNAs are localized to axons, regardless of how they got here. If they authors want to keep the figures as part of this manuscript, they should test whether transport dynamics are affected by Semaphorin3A treatment, and they should identify the vesicles further using specific markers for LE, lysosomes, MVBs, etc.

AUTHORS’ REPLY: As mentioned above in our answer to point 5, we have identified the vesicles further using markers for early and late endosomes / lysosomes. Our results and main conclusions are detailed in the answer to the Editor, point 1 above in page 1-2.

We have also tested whether transport dynamics is affected by Sema3A treatment and found that Sema3A elicits a change in puncta distribution per speed category without altering the overall speed of individual puncta and their directionality. Specifically, Sema3A induces an increase in the number of static puncta (<0.2μm/s) and a decrease in the number of fast moving puncta (>0.5μm/s). This effect appears to be specific to Sema3A as neither Netrin-1 nor Slit2 perturb pre-miR-181a-1 axonal trafficking. Since Slit2 does not lead to pre-miR-181-a processing, it is tempting to speculate that Sema3A regulation of miRNA precursor processing and trafficking are coupled. By increasing the number of static puncta at the expense of fast moving ones, Sema3A would in effect reduce the number of pre-miRNA molecules reaching and leaving the growth cones. Overall, Sema3A would induce the production of NGmiRNAs but this production would cease when the local supply of precursor runs out and is not replenished due to decreased trafficking. This would result in a discrete, self-limiting burst of miRNA production, ensuring that only a specific number of pre-miRNAs are processed in response to cue to maintain the stoichiometry between mature miRNA and mRNA target required to induce appropriate silencing.

These results are now included in supplemental material. We have modified the figure (Fig EV3), results (page 9 and 10 (lines 244-249), and discussion (page 16 and 17 (lines 426-434) accordingly.

7.Line 179 related to Fig 4D: The HA- and neurofilament signal overlap only very little. Most if not all HA-Dicer seems to be outside of axons. A higher magnification and co-localization analysis would be necessary to support the authors' claim of clear overlapping signals.

AUTHORS' REPLY: We have now produced additional pictures and performed colocalization analysis.

In our study, we used neurofilament marker to be able to distinguish the most superficial region of the superior colliculus, the stratum griseum superficiale (SGS), enriched in RGC axons (Ito S. and Fedlheim 2018, Front. Neural Circuits). Our new additional images display the stereotypical structure of the superior colliculus and show a neurofilament marked-SGS subregion clearly devoid of nuclei and containing a clear HA-positive signal (corresponding to Dicer). These new images can be found in Fig EV2.

Additionally, we have performed colocalization analysis by computing Manders coefficient and associated Costes' randomization test and found a low but significant 22% colocalization between Dicer-HA and neurofilament in the superior colliculus SGS (see below for details).

It is important to specify that we do not expect a high co-colocalization between Dicer-HA- and neurofilament-derived signals. Each neurofilament (0.2-0.3 µm wide in our pictures) does not strictly correspond to one single axon (up to 3.5 µm, Perge et al., 2009, J Neurosci)(see cross section below). Within an axon, neurofilaments are distributed as long numerous individual filaments interspaced by microtubules (MT) (e.g. Pan and Chan, 2017, JCB). In light of this, we expect Dicer to be also located in-between neurofilaments of a single axon, perhaps preferentially associated with MT for transport. This is indeed what we observe (see illustrative figure below), as Dicer-associated signal appears to be located adjacent and partly overlapping with, or inbetween Neurofilament-associated signal. The significant 22% colocalization that we measure between Dicer-HA and neurofilament is consistent with this observation.

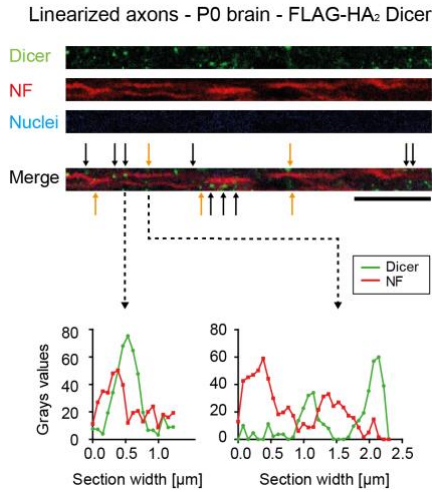


Figure A: Signal colocalization across an axon. Upper panel: Linearized RGC axon from P0 FLAG-HA2-Dicer mice depicting Dicer and neurofilaments (NF) signals in a free nuclei region. Overlapping Dicer and NF signal (black arrows), juxtaposition signal (orange arrows). Lower panel: Intensity plots of Dicer and NF overlapping signal (left), and of Dicer peaks in-between two neurofilaments (right). Abbreviations: P0, post-natal day 0, NF, neurofilament. Scale bars: 10µm.

Details about the colocalization analysis:

As mentioned above, the proportion of Dicer signal colocalizing with neurofilament was assessed by computing Manders' coefficient (M1) (Manders et al, 1993, J. Microsc.). M1 considers the signal derived from channel 1 (here, Dicer-HA positive-signal) as co-localized with the signal derived from a thresholded channel 2 (here neurofilament positive signal) when both signal overlaps. This overlap is calculated after applying a predefined threshold of pixel intensity to channel 2 automatically computed relying on spatial statistics (Costes et al, 2004, Biophysical journal). As aforementioned, this colocalization analysis revealed that 22.23% +/- 1.11 of Dicer overlaps with neurofilament within the superior colliculus SGS of Dicer-HA KI mice (Fig.A above). The measured co-localization was subsequently tested for significance with Costes' randomization test (*Costes et al. 2004*, Biophysical journal). Costes' test computes the probability (P-value) that the correlation between the two channels of the acquired image is significantly greater than the one obtained by random overlap. This is achieved by scrambling pixel blocks of channel 2 image, and then measuring the correlation with the real channel 1 image. The scrambling and measuring overlap of the two signals was repeated 100 times for each analysed region. Co-localization was considered significant when Costes' p-value > 0.95, that is when less than 5% of the randomizations produced a higher correlation than the value measured in the acquired image. The Costes's p-value was equal to 1 in all the analysed z-layers, meaning that out of the 100 randomized images none showed better correlation between Dicer and neurofilaments than the original image.

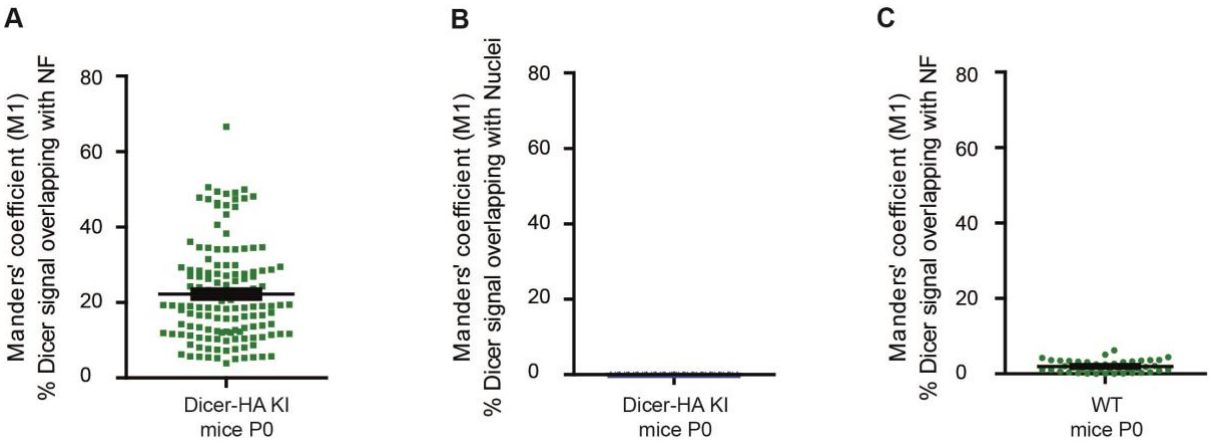
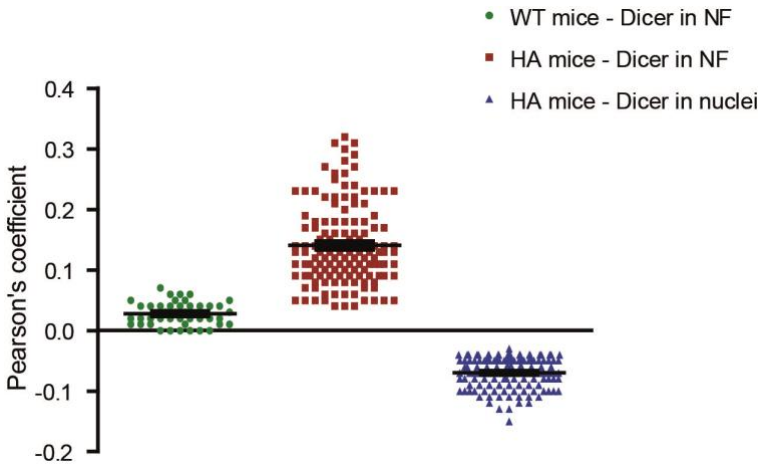


Figure B: Mender's coefficient co-localization analysis. % of Dicer signal in RGC projection overlapping with either Neurofilaments (NF) or Nuclei, in Dicer-KI (A, B) or WT P0 mice (C). Each dot corresponds to a single focal plane (0.345 µm) of RGC projection, n=136 (Dicer-KI mice) and n=43 (WT mice). Data from 3 Dicer-KI mice P0, and 2 WT mice P0.

As a negative control for the co-localization analysis pipeline, we assessed whether Dicer was absent from nuclei as previously reported (Much et al. 2016, PLOS Genetics). We found a 0% signal overlap between of Dicer-HA and To-Pro nuclear staining in sagittal brain sections of P0 mice (Fig.B). Costes's randomization test confirmed that colocalization was not significant. Indeed, it revealed that 100% of the random images showed a better correlation than the real image. Finally, we performed a similar analysis on WT mice as an additional negative control and

found a 1.97 % +/- 0.23 non-significant overlap between Dicer and neurofilament (Fig.C).

We finally computed Pearson's coefficient on the same samples (see below). As expected, we calculated a positive correlation between Dicer-HA and neurofilament (0.1406 +/- 0.0056) in HA-KI mice, no correlation in WT mice (0.0276 +/- 0.0029) and a negative correlation between Dicer-HA and nuclei TO-PRO marker (-0.0697 +/- 0.0021).



8.Lines 193-197 related to Figs. 5B,C: the authors should check for mir-182-5p and -3p levels as negative controls.

AUTHORS' REPLY: Following the Reviewer's suggestion, we have measured the levels of mature miR-182-5p and miR-182-3p in isolated axons upon Sema3A stimulation. We find that Sema3A does not alter the levels of these mature miRNAs. We have already shown in a previous publication that Slit2 stimulation does not alter miR-182-5p levels using RT-qPCR on pure axons (see Bellon et al., 2017, Cell reports). In light of this and also since miR-182-3p is lowly expressed in axons and 15.9 fold less abundant than miR-182-5p in this compartment, we didn't test whether miR-182-3p levels change upon Slit2 stimulation.

We have modified the figure (Appendix Fig S4D,E), results (page 9 (lines 232-235) to incorporate this new control.

9.Lines 251-253: The argument against mRNA degradation based on the time frame is weak. The authors should measure target mRNA levels by qRT-PCR if they want to make this claim.

AUTHORS' REPLY: We have now removed from page 11 (line 302) our argument against mRNA degradation based on the time frame.

10.Figure 7: This entire figure is using overexpressed reporter constructs as proxies for the translation of endogenous mRNA. The authors' main finding of the paper is that local pre-miRNA processing shifts the balance between mRNA and miRNAs locally (see scheme). The overexpression of the reporter constructs is likely to affect this balance as well, confounding the interpretation of these data. Instead of using exogenous report constructs, it would be much stronger and more reliable to measure the translation of endogenous target mRNAs of miR-181a directly. There are several methods such as puro-PLA or BONCAT that can be used together with the MOs.

AUTHORS' REPLY: Following the Reviewer's suggestion, we have assessed whether translation of endogenous TUBB3 mRNA is altered by Sema3A in isolated axons using puro-PLA. We find that Sema3A exposure significantly reduced the levels of newly synthesized TUBB3 specifically in growth cones compared to PBS control. We further tested whether NGmiRNAs modulate Sema3A-induced silencing of endogenous TUBB3 using Dicer cleavage MOs ("MOs-3p"). We obtained similar results as the ones generated with our reporter. When pre-miR-181a-1 processing was blocked with MOs-3p, we detected that Sema3A was no longer able to elicit endogenous TUBB3 silencing. Overall, these new data confirm the results obtained using Venus-TUBB3 3'UTR as an exogenous translational reporter.

Furthermore, the rescue experiments requested in the point below also suggests that endogenous, miRNA-dependent TUBB3 mRNA translational silencing is induced by Sema3A signaling cascade. Indeed, when we specifically block the translation of TUBB3 in isolated axons in a miR-181 morphant background, growth cone sensitivity to Sema3A is restored.

Together, these three sets of experiments enable us to confidently conclude that endogenous TUBB3 is regulated by the Sema3A-NGmiR-181 axis in axons.

We have created a new figure (Fig 9) and described these results in a new section entitled "Endogenous TUBB3 is a critical target of NGmiRNAs in growth cone steering" in page 14 (358-372).

11.How many localized mRNAs are likely regulated by miR-181a? Does delivery of siRNA against TUBB3 rescue the growth cone collapse phenotype seen in Fig. 5E,F or in other words?

AUTHORS' REPLY: The Reviewer is rising interesting questions. In the present study, we performed total RNA sequencing from isolated axons and detected that 526 axonal mRNAs are putative miR-181a-5p targets according to specific selection criteria (targets were conserved in mouse and human and ranked amongst the 20% best candidates according to TargetScan ContextScore). We cannot give a precise estimate of how many mRNAs are likely regulated by miR181a, since not

all putative targets are *bone fide* targets (miRNA target prediction is prone to many false positives). Indeed we find that THBS1 and APP, two of our strongest candidates, were not regulated by miR-181 at basal levels or upon Sema3A stimulation. It is possible that cues other than Sema3A may elicit miRNA-mediated regulation of these mRNAs. Alternatively, these putative targets might not be readily accessible to the miRNA for silencing, for instance due to the competitive binding of an RBP in the vicinity of the MRE within the 3'UTR (Krol et al., 2010, Nat Rev Genet). Regardless, we have now specified in the text in page 43 (line 1103), how many predicted targets we detected.

Furthermore, we have addressed whether knocking down TUBB3 rescues the growth cone collapse phenotype seen in Fig. 5F (new Fig 6F). We find that cotransfection of axons with TUBB3 MO and miR-181 MOs-3p fully rescues Sema3A-induced collapse response. This suggests that TUBB3 is a key mediator of miR-181 in inducing Sema3A-mediated collapse.

As mentioned above, we have created a new figure (Fig 9) and described these results in a new section entitled "Endogenous TUBB3 is a critical target of NGmiRNAs in growth cone steering" in page 14 (line 368-372).

Minor Points:

12.Lines 23, 58 - what is the meaning of delocalization? From the context it should be localization.

13.Line 49 - use 'and' instead of 'but', as the sentence does not state a contradiction.

14.Line 88 - reformulate half-sentence following 'and'.

15.Line 136 - remove promptly.

16.Line 160 - Spelling: co-traffic.

17.Line 207 - 6A should be S6A.

AUTHORS' REPLY: We apologize for the mistakes. All these points have been addressed and the manuscript adjusted accordingly (see yellow highlights at the corresponding lines).

12. page1 lines 28 and page 2 line 63
13. page 2; line 55
14. page 3; line 91
15. page 5; line 145
16. page 7; line 173
17. page 10; line 257

Reviewer #3:

The study emerged from earlier intriguing findings that besides mRNAs also other RNAs like non-coding RNAs, miRNAs etc are also found enriched in specific subcellular outposts, but the underlying mechanism remained elusive until now. The authors first convincingly show, using optimized procedures with molecular beacons, that precursor pre-miRNAs appear to be actively transported to the growth cone central area by hitchhiking on CD63-positive late endosomal organelles along the axons. These findings extend a recent study where mRNAs use endosomal compartments for transport and as organellar translation hubs. Next, they demonstrate that in the growth cone, these pre-miRNAs are processed into active miRNAs and that this occurs in a response to repressive cues. Finally, they identify one of the targets, TUBB3, that is silenced through specific miRNAs when neurons are exposed to Sema3A. Overall, the authors describe a novel mechanism by which neuronal outgrowth and remodelling can be modulated through local activation of specific miRNAs in response to cues. Moreover, they provide important in vivo support for this mechanism, upkeeping a new additional regulatory layer in brain connectivity. Thus, external cues can activate parallel pathways regulating the expression of separate growth cone proteins, on through eliciting a local increase in protein synthesis, while another one inhibits protein synthesis (as an alternative to local protein degradation). This allows for a fine-tuned balance in axon remodelling during brain development.
The experiments, both in vitro and in vivo, are to my opinion well-controlled and convincing. The authors did overcome several technical hurdles to uniquely detect premiRNAs, implemented a broad range of approaches and controls that brought together an impressive set of original data. To my opinion, the originality and wealth of data support publication in EMBO Journal.
I do not have major concerns, but rather some reflections that may require some more explanation or control experiments.

AUTHORS' REPLY: We thank the Reviewer for the insightful comments and criticisms that contributed to improve the quality of our work, and for pointing out that "The experiments [are] well-controlled and convincing", that our work "brought together an impressive set of original data" and that the "originality and wealth of data" contained in our manuscript.

Minor concerns:

1 - PremiRNAs traffick retrogradely and anterogradely with the same frequencies. Why are premiRNAs retrogradely transported and is there like the central region in the growth cone also a region in the retrograde direction (cell body?) where they concentrate (and may become activated as well)?

AUTHORS' REPLY: We thank the Reviewer for these comments also raised by the other Reviewers. We have addressed thoroughly the potential physiological impacts of pre-miRNA retrograde transport in the reply to the Editor (point No2, page 2 and 3) and summarized our key points in the discussion on page 17 (lines 435-446).

To investigate whether pre-miRNAs accumulates outside of the growth cone, we dissociated ocular explants after electroporation and, intriguingly, noticed that pre-miR-181a-1 concentrated in the perinuclear region of the RGC cell body where CD63 markers also gathered. Figure 3H and I and results on page 7 (line 177) have been modified accordingly.

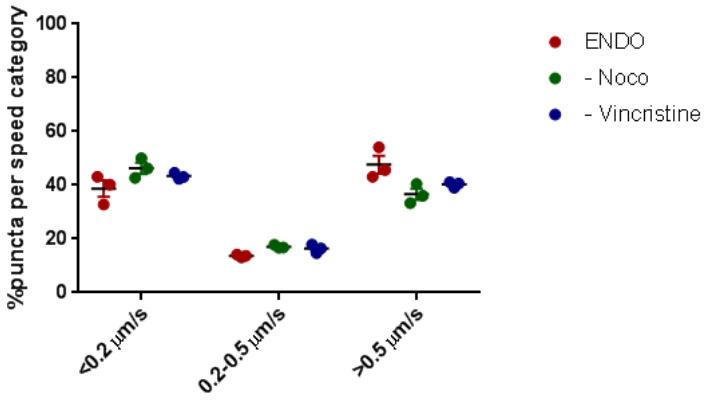
2 - Fig. 3B,C: treatment with nocodazole increases the proportion of stationary puncta while an overall decrease of average velocities of puncta. When comparing this set of data with figure 2, the proportion of actively moving puncta is by far the major population in panel E and H, while in fig 3, it is roughly similar in proportion to the confined puncta (red bars in fig 3C). Do the authors have an explanation for this as they claim that most premiRNA are actively transported along MTs.

AUTHORS' REPLY: The analyses depicted in Figure 2 and Figure 3 are not the same which would explain why the data distribution appear different. For this reason, they cannot be formally compared.

- Fig 2E vs former Fig 3C (new Fig 2K). Fig 2E shows average velocity in µm/s while 3C (new Fig 2K) shows the percentage of puncta per speed category.
- Fig 2H vs Fig 3C (new Fig 2K). Fig. 2H plots the MSD α coefficient of only moving particles (with a speed > 0.2µm/s). The coefficient α is derived from the following equation $MSD=A\tau_{\alpha} +B$ and is an indication of the particle motion-type (Otero et al, 2014), with $\alpha>1.5$, actively driven; $0.9<\alpha<1.1$, diffusive; $\alpha<0.5$, confined. Former Fig. 3C (new Fig 2K) on the other hand, plots the percentage of puncta per speed category, not by alpha category, and include <0.2µm/s but also 0.2-0.5µm/s and >0.5µm/s.

To be able to compare the distribution speed generated in Fig 2 to the one generated in former Fig. 3C (new Fig. 2K), we display below the percentage of puncta per speed category for endogenous pre-miR-181a-1 and for Nocodazole controls ("-Noco"). We have also plotted the distribution for our new Vincristine controls

(Appendix Fig EV1E). Displayed data are mean +/- SEM. Each data point corresponds to one independent experiment.



3 - Related to figure 3 the authors indicate that mRNAs are trafficked along axons within RNPs, while data indicate that miRNAs (and other related RNAs) associate with LE/MVBs. Therefore, the authors reasoned that premiRNAs may use LE/MVBs to traffick along axons. However, recently (Cione et al., 2019) mRNAs were found to be associated with endosomal organelles (not limited to LE/MVBs) as well during transport. It would be interesting to demonstrate whether the same or distinct populations of LE/MVBs are used for both cargo's. Likewise, the authors cannot exclude that other organelles besides LE/MVBs are involved: a co-staining with other endosomal organelles (EEA1-/Rab5-GFP, Lamp1-GFP) should be performed to demonstrate unique associations.

AUTHORS' REPLY: We have further characterized the populations of LE/MVBs to which pre-miRNA associate. In Cioni et al., 2019, Rab7a, Rab5a, Lamp1 and LysoTracker were used as markers, which we have also employed here. Our complete analysis is detailed in our answer to the Editor 's first point above on page 1-2. Collectively, these data suggest that pre-miRNAs are primarily transported by late endosomes / lysosomes. One major difference in the conclusions of our study and that of Cioni et al. is that pre-miRNAs hitchhike onto endosomes (our study) whilst mRNA associate with endosomes for translation but not trafficking (Cioni) therefore we do not expect TUBB3 mRNA to be located on trafficking endosomes with pre-miRNAs.

4. To conclude the authors show that in the growth cone Sema3A induces an activation cascade generating local miRNAs that silence specific targets such as TUBB3 resulting in MT remodelling and finally growth cone collapse. The immediate next and important question is how Sema3A signals to the machinery activating local premiRNAs. Although this requires a new set of experiments beyond the scope of the current manuscript, can the authors speculate a bit more and include their thoughts in the discussion section?

AUTHORS' REPLY: We agree with the Reviewer that addressing how Sema3A signals to the machinery activating local premiRNAs is the immediate next and important question. We envision that Sema3A would employ similar signaling cascade as the one modulating the translation of known transcripts. As developed below, it is tempting to speculate that Sema3A-induced activation of Neuropilin-1/Plexin-A receptor complex would trigger ERK1/2 activation which in turn would activate Dicer cofactors and pre-miRNA processing. Alternatively pre-miRNA processing could be blocked in basal conditions by an RBP that masks pre-miRNA Dicer cleavage site. In this second case scenario, ERK1/2 activation would elicit the phosphorylation of this RBP, thereby releasing and exposing Dicer cleavage site for processing. The discussion of the revised manuscript has been adjusted accordingly on page 20 (lines 508-524).

Sema3A collapse response relies on protein synthesis in *Xenopus* RGC axons through the following signaling cascade (Campbell, D. S et al. 2001, Neuron). Sema3A is recognized by a receptor complex formed by Neuropilin-1 (NP1) and Plexin-A (PlexA) (Rohm B. et al. 2000, Mechanisms of Development) which elicits MAPK p42/p44 (ERK1/2) activation and local protein synthesis through mTOR (Campbell, D. S et al. 2001, Neuron). Here we show that in parallel to mRNA translation induction, specific basally translated mRNAs are repressed by Sema3A via NGmiRNAs. From this, we surmise that Sema3A-induced pre-miRNAs processing could be mediated by the same ERK1/2-based signalling pathway.

As proposed above, ERK1/2 activation might lead to Dicer activation and concomitant processing of pre-miRNAs. We observe that only a few specific pre-miRNAs are processed upon Sema3A exposure. Importantly, activation of given Dicer cofactors, such as TRBP, confer specificity to the Dicer complex (Pullagura, Sri Ramulu N et al. 2018, Genetics). TRBP phosphorylation increases Dicer activity by stabilizing the miRNA generating complex (Paroo, Zain et al. 2009, Cell). Intriguingly in neuronal progenitor cells, TRBP becomes phosphorylated upon ERK activation and promotes miR-181a maturation (Xu, Chi et al. 2015, Stem cells). It is therefore possible that in RGC axons Sema3A activates ERK pathway, leading in turn to the phosphorylation/activation of TRBP and pre-miR-181a-1/2 processing.

Alternatively, Sema3A-induced ERK signaling might lead to the specific processing of pre-miRNA via RNA-binding protein (RBP) phosphorylation and subsequent exposure of pre-miRNA Dicer cleavage site. The binding affinity of specific RBPs for their target RNAs can change depending on their phosphorylation status (Venigalla and Turner, 2012, Front Immunol; Darnell, 2013, Annu Rev Neurosci.) Furthermore, RBP can compete with Dicer to hinder pre-miRNA processing (Loffreda et al., 2015, Biomolecules). It is thus possible that pre-miR-181a-1 is bound by a specific RBP thereby preventing Dicer from accessing its cleavage site. Upon activation of Sema3A-induced ERK signaling cascade, this RBP would be phosphorylated and release its target. Pre-miRNA would thereby be freely accessible for Dicer cleavage. The binding and signaling-induced release of particular RBP from pre-miRNA targets would confer specificity to the pre-miRNA processing.

2nd Editorial Decision 4th Dec 19

Thank you for submitting your revised manuscript for our consideration, it has now been seen once more by the original referees (see comments below). I am pleased to say that the referees overall find that their initial concerns have been satisfactorily addressed and now support publication. Referee #3 raises one more point that can however be addressed in a final revised version of the manuscript. In addition, I would also ask you to take care of several editorial issues that are listed in detail below. Please make any changes to the manuscript text in the attached document using the "track changes" option. Once these minor issues are resolved, we will be happy to formally accept the manuscript for publication.

REFEREE REPORTS

Referee #1:

I found the revised version of this manuscript greatly improved and addressing the main criticisms raised by the reviewers. Whilst not all the points have been addressed, the findings summarised in this manuscript and their potential impact now warrant publication.

Referee #2:

The authors have answered all original questions and I have no further concerns.

Referee #3:

The authors have thoroughly revised their manuscript and adequately addressed my concerns. I have taken the time to also look into the other comments and feel the authors have addressed these as well with additional experiments and valuable controls.
There is only one small remark with respect to the discussion on the relevance of retrograde trafficking of pre-miRNAs. The authors state that: "Pre-miRNAs may be scanning the axons until they are required for mRNA silencing at t he growth conecone and perhaps also along the axon where local translation has be en reported to occur." As pre-miRNA hitchhike on LE/Lys, it is likely the LE/Lys that defines the balance between retro- and anterograde trafficking. If correct, the authors suggest that the LE/Lys has the intrinsic feature and machinery to 'scan the axon' and locate areas where local translation is needed. This is a conclusion that may be too farfetched on the basis of the data and I would suggest to more carefully address this.

2nd Revision - authors' response 24th Dec 19

Referee #3:

The authors have thoroughly revised their manuscript and adequately addressed my concerns. I have taken the time to also look into the other comments and feel the authors have addressed these as well with additional experiments and valuable controls.
There is only one small remark with respect to the discussion on the relevance of retrograde trafficking of pre-miRNAs. The authors state that: "Pre-miRNAs may be scanning the axons until they are required for mRNA silencing at t he growth conecone and perhaps also along the axon where local translation has be en reported to occur." As pre-miRNA hitchhike on LE/Lys, it is likely the LE/Lys that defines the balance between retro- and anterograde trafficking. If correct, the authors suggest that the LE/Lys has the intrinsic feature and machinery to 'scan the axon' and locate areas where local translation is needed. This is a conclusion that may be too farfetched on the basis of the data and I would suggest to more carefully address this.

REPLY OF THE AUTHORS: We thank the reviewers for pointing out the fact that our conclusion might be too farfetched. We have thus modified the discussion accordingly and removed from the text the speculative sentence suggesting that “LE/Lycan locate areas within the axon where local translation is needed”

Accepted 17th Jan 20

Thank you again for submitting the final revised version of your manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Marie-Laure Baudet
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2019-102513R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical approaches were implemented to define the sample sizes. Sample sizes were choosen accordengly to what is generally used in our research field (e.g. live imaging, FRAP) or those that were routinely used previously with analogous methodological approaches (e.g. collapse assay, RT-qPCR)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Each independent experiment was run using embryos from the same fertilized frog. Number of explants and/or axons for each experiment was choosen based on previous experiments with similar methods.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All the criteria of axonal selection for the live imaging are described in the Material and Methods section. For the behavioural assay (Fig. 6h-i) serial electroporation in both eyes were applied. A pre-established criteria for embryos exclusion was brain contamination in the electroporation: if cells different from RGC primordia were targeted, embryos were not tested. One control and two miR-181-MO embryos data were excluded because of too high electroporation levels which might be affected the more physiological vision read-out of the assay.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The order of samples processing as well as drug/stimulation treatments were randomized and differ among different experiments.
For animal studies, include a statement about randomization even if no randomization was used.	For Mus Musculus experiments, animals were randomly selected from the litter. For ex vivo experiments with Xenopus laevis embryos only healthy animal were selected for any experiments. Healthy state of embryos and/or axons was checked before any experiments or drugs application. Unhealthy embryos/axons were not used for doing experiments, as well as embryos from in vitro feritilization with a low survival rate.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Collapsed assays were blindly counted. RGC culture plates were randomly allocated for the different experimental conditions.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For animal studies (e.g. Xenopus laevis behavioral assay) no blinding was applied.
5. For every figure, are statistical tests justified as appropriate?	A stat table is included in the supplement material, reporting all details for each Figure. The test used and the sample size is reported also in Figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normality distribution was assessed with Graph Pad Prism, and the statistical test applied was choosen accordingly. Both Figure legend and stat table report the result of normality test.
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	No F-test was run to statistically compare the variance.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-report	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used: 1) anti-Rabbit, Alexa Fluor 488 conjugated Life technologies Cat# A11070; 2) anti-Mouse, Alexa Fluor 594 conjugated Life technologies Cat# A11020; 3) anti-Rabbit, Alexa Fluor 647 conjugated Life technologies Cat# A21246; 4) Dicer Antibody (H-212) Santa Cruz Biotechnology Cat# sc-30226; 5) Neurofilament-associated antigen antibody Developmental Studies Hybridoma Bank Cat#3A10-c; 6) HA-probe antibody (Y-11) Santa Cruz Biotechnology Cat# sc-805; 7) Mouse monoclonal anti-Ago2 Sigma-Aldrich Cat#SAB4200085; 8) Rabbit-anti-beta-Tubulin III, Sigma-Aldrich Cat#T2200; 9) Rabbit-anti-Rab7a Abcam Cat# 137029; 10) Anti-Puromycin Antibody, clone 12D10, Merck Millipore, Cat#MABE343. Validation: Dicer Antibody (H-212) Santa Cruz Biotechnology Cat# sc-30226. This primary antibody has been recently used in Nature Communications Zhang C et al. 2018 (in mice) and in Nature Powers JT et al. 2016 (mice). In the webpage of the product the reactivity is garanteed for human and mice. For the epitope used, 1701-1912 aa of the human sequence, the identity with Xenopus is 99% (Xenbase sequence and multiple alignment). Neurofilament-associated antigen antibody Developmental Studies Hybridoma Bank Cat#3A10-c. In the webpage of the product, mouse is listed among the positive tested species reactivity, used in this paper as a general counterstaining for axons. HA-probe antibody (Y-11) Santa Cruz Biotechnology Cat# sc-805. In this paper, WT mice which do not have and HA tag for Dicer, and are negative for the staining with sc-805 primary antibody. Anti-Rab7 has been already used in Xenopus (Cioni JM et al. 2019). Validated by Abcam, used in 24 published scientific works (https://www.abcam.com/rab7-antibody-epr7589-ab137029-references.html#top-1064). Anti-puro has been already used in Xenopus (Cioni et al. 2019) for PLA protocol, as we did. Anti-tubb3 was validated by Western by the company (<https://www.sigmaaldrich.com/catalog/product/sigma/t2200?lang=it&region=IT>) and previously used in Xenopus (Lin G et al. 2007) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1890292/ and (Livigni A et al. 2013) https://www.ncbi.nlm.nih.gov/pubmed/24210613
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D-

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Xenopus laevis were bought from Nasco; Mus Musculus Flag-HA2-Dicer C57BL6 mice (Comazzetto et al, 2014) were kindly donated by Dónal O'Carroll. Xenopus laevis embryos were obtained by in vitro fertilization, raised in 0.1x MMR, pH 7.5 at 14-22°C and staged according to (Nieuwkoop & Faber, 1994). Mice were housed and maintained, in accordance with the Decreto Legislativo 4 marzo 2014, n°26 in the animal facility at Di.CIBIO (Trento, Italy). For both animal models only embryos were used for experiments: from st4 (8-cells) to st44 for Xenopus laevis; E13.5 and P0 for mice.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by the University of Trento Ethical Review Committee and by the Italian "Ministero della Salute" both according to the D.Lgs nr.116/92 and with the authorization n°1159/2016-PR and n°546/2017-PR according to art.31 of D.lgs. 26/2014.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed

E-

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F-

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Done
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	The publicly available miRNA-sequencing dataset is deposited in GEO (GEO: GSE86883): Bellon, A. et al. miR-182 Regulates Slit2-Mediated Axon Guidance by Modulating the Local Translation of a Specific mRNA. Cell Rep 18, 1171–1186 (2017). The RNA-sequencing data generated in this study will be deposited in GEO (GEO accession number). The data that support the findings of this study are available from the corresponding author upon request.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	All software and algorithms used for computational analysis are reported in Supplement Table 4 (accession numbers and links are included).

G-

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	The study in this paper does not fall under dual research restriction.